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PATENT APPLICATION
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OPTIMAL WINDOWS FOR OBTAINING OPTICAL DATA FOR CHARACTERIZATION OF TISSUE SAMPLES

Prior Applications

[0001] The present application claims the benefit of U.S. Provisional Patent Application Serial Number 60/394,696, filed July 9, 2002, which is hereby incorporated by reference.

Field of the Invention

[0002] The invention relates generally to spectroscopic methods. More particularly, the invention relates to the diagnosis of disease in tissue using spectral analysis and/or image analysis.

Background of the Invention

[0003] Spectral analysis is used to diagnose disease in tissue. For example, data from spectral scans performed on the tissue of a patient are used to screen tissue for disease. Some diagnostic procedures include the application of a chemical contrast agent to the tissue in order to enhance the image and/or spectral response of the tissue for diagnosis. In an acetowhitening procedure, acetic acid is used as the contrast agent. Use of a contrast agent enhances the difference between data obtained from normal tissue and data obtained from abnormal or diseased tissue.

[0004] Current techniques do not suggest an optimal time period following application of a contrast agent within which to obtain spectral and/or image data for the diagnosis of disease, nor do current techniques suggest how such an optimal time period could be determined.

Summary of the Invention

[0005] The invention provides optimal criteria for selecting spectral and/or image data from tissue that has been treated with a contrast agent for disease screening. In particular, it has been discovered that the sensitivity and specificity of optical diagnostic screening is improved by obtaining optical data at optimal time points after application of a contrast agent.

[0006] Accordingly, methods of the invention provide optimal windows in time for obtaining spectral data from tissue that has been treated with a contrast agent in order to improve the results of disease screening. The invention further provides methods for identifying such windows in the context of any optical diagnostic screen. Additionally, the invention provides methods for disease screening using kinetic data obtained across multiple diagnostic windows. Methods of the invention allow an optical diagnostic test to focus on data that will produce the highest diagnostic sensitivity and specificity with respect to the tissue being examined. Thus, the invention allows the identification of specific points in time after treatment of a tissue when spectral and/or image data most accurately reflects the health of the tissue being measured.

[0007] Time windows for observing selected spectral data may be determined empirically or from a database of known tissue responses to optical stimulation. For example, in one aspect the invention comprises building and using classification models to characterize the state of health of an unknown tissue sample from which optical signals are obtained. As used

herein, an optical signal may comprise a discrete or continuous electromagnetic signal or any portion thereof, or the data representing such a signal. Essentially, optical diagnostic windows are based upon the points at which classification models perform best. In practice, optimal diagnostic windows of the invention may be predetermined segments of time following application of a contrast agent to a tissue. Optimal diagnostic windows may also be points in time at which an optical measurement meets a predetermined threshold or falls within a predetermined range, where the optical measurement represents the change of an optical signal received from the tissue following application of a contrast agent. For example, a window may be selected to include points in time at which the change in optical signal intensity from an initial condition is maximized. Finally, the optical measurement upon which a window is based may also reflect the rate of change in a spectral property obtained from the tissue.

[0008] In a preferred embodiment, optimal windows are determined by obtaining optical signals from reference tissue samples with known states of health at various times following application of a contrast agent. For example, one embodiment comprises obtaining a first set of optical signals from tissue samples having a known disease state, such as CIN 2/3 (grades 2 and/or 3 cervical intraepithelial neoplasia); obtaining a second set of optical signals from tissue samples having a different state of health, such as non-diseased; and categorizing each optical signal into "bins" according to the time it was obtained in relation to the time of application of contrast agent. The optical signal may comprise, for example, a reflectance spectrum, a fluorescence spectrum, a video image intensity signal, or any combination of these.

[0009] A measure of the difference between the optical signals associated with the two types of tissue is then obtained, for example, by determining a mean signal as a function of wavelength for each of the two types of tissue samples for each time bin, and using a discrimination function to determine a weighted measure of difference between the two mean optical signals obtained within a given time bin. This provides a measure of the difference between the mean optical signals of the two categories of tissue samples – diseased and healthy – weighted by the variance between optical signals of samples within each of the two categories.

[0010] In one embodiment, the invention further comprises developing a classification model for each time bin. After determining a measure of difference between the tissue types in each bin, an optimal window of time for differentiating between tissue types is determined by identifying at least one bin in which the measure of difference between the two tissue types is substantially maximized. For example, an optimal window of time may be chosen to include every time bin in which the respective classification model provides an accuracy of 70% or greater. Here, the optimal window describes a period of time following application of a contrast agent in which an optical signal can be obtained for purposes of classifying the state of health of the tissue sample with an accuracy of at least 70%.

[0011] An analogous embodiment comprises determining an optimal threshold or range of a measure of change of an optical signal to use in obtaining (or triggering the acquisition of) the same or a different signal for predicting the state of health of the sample. Instead of determining a specific, fixed window of time, this embodiment includes determining an optimal threshold of change in a signal, such as a video image whiteness intensity signal, after which an optical signal, such as a diffuse reflectance spectrum and/or a fluorescence

spectrum, can be obtained to accurately characterize the state of health or other characteristic of the sample. An embodiment includes monitoring reflectance and/or fluorescence at a single or multiple wavelength(s), and upon reaching a threshold change from the initial condition, obtaining a full reflectance and/or fluorescence spectrum for use in diagnosing the region of tissue. This method allows for reduced data retrieval and monitoring since, in an embodiment, it involves continuous tracking of a single, partial-spectrum or discrete-wavelength "trigger" signal (instead of multiple, full-spectrum scans), followed by the acquisition of one or more spectral scans for use in diagnosis. Alternatively, the trigger may include more than one discrete-wavelength or partial-spectrum signal. The diagnostic data obtained will generally be more extensive than the trigger signal, and may include one or more complete sets of spectral data. The measure of change used to trigger obtaining one or more optical signals for tissue classification may be a weighted measure, and/or it may be a combination of measures of change of more than one signal. The signal(s) used for tissue classification/diagnosis may comprise one or more reflectance, fluorescence, and/or video signals. In one embodiment, two reflectance signals are obtained from the same region in order to provide a redundant signal for use when one reflectance signal is adversely affected by an artifact such as glare or shadow. Use of multiple types of classification signals may provide improved diagnostic accuracy over the use of a single type of signal. In one embodiment, a reflectance, fluorescence, and a video signal from a region of a tissue sample are all used in the classification of the region.

[0012] In a further embodiment, instead of determining an optimal threshold or range of a measure of change of an optical signal, an optimal threshold or range of a measure of the rate of change of an optical signal is determined. For example, the rate of change of reflectance

and/or fluorescence is monitored at a single or multiple wavelength(s), and upon reaching a threshold rate of change, a full reflectance spectrum and/or fluorescence spectrum is acquired for use in diagnosing the region of tissue. The measure of rate of change used to trigger obtaining one or more optical signals for tissue classification may be a weighted measure, and/or it may be combination of measures of change of more than one signal. For example, the measured rate of change may be weighted by an initial signal intensity.

[0013] The invention also provides methods of disease screening using kinetic data from optical signals obtained at various times following application of a contrast agent. These methods comprise techniques for using specific features of fluorescence and diffuse reflectance spectra from reference cervical tissue samples of known states of health in order to diagnose a region of a tissue sample. These techniques allow monitoring of a particular optical signal from a test sample during a specified period of time following application of contrast agent to obtain pertinent kinetic data for characterizing the sample. For example, two or more time-separated measures of video intensity, fluorescence, and/or reflectance are obtained for a test sample at times between which it is known that an increase or decrease indicative of a given state of health occurs. It is therefore possible to determine whether this increase or decrease has occurred for the test sample, thereby indicating the sample may have a given state of health. Alternatively or additionally, a video, reflectance, and/or fluorescence signal from a test sample may be monitored over time to determine a time at which the signal reaches a maximum or minimum value. The time following application of contrast agent at which this minimum or maximum is reached can then be used to determine indication of a disease state in the test sample.

[0014] In one embodiment, data used as a baseline in determining an increase, decrease, maximum, or minimum as discussed above is not obtained before, but is obtained immediately following application of contrast agent to the tissue. In one case, the time period immediately following application of contrast agent is about ten seconds, and in another case, it is about five seconds, although other time periods are possible. This may be done to avoid error caused by movement of tissue or movement of the optical signal detection device upon application of contrast agent, particularly where such movement is not otherwise compensated for. Movement of tissue may cause error where a change from an initial condition is being monitored and the region of the tissue corresponding to the location at which the initial signal was obtained shifts following application of contrast agent.

Brief Description of the Drawings

[0015] The objects and features of the invention can be better understood with reference to the drawings described below, and the claims. The drawings are not necessarily to scale, emphasis instead generally being placed upon illustrating the principles of the invention. In the drawings, like numerals are used to indicate like parts throughout the various views.

[0016] Figure 1A shows a graph depicting mean fluorescence spectra before application of acetic acid and at various times following the application of acetic acid for NED tissue (no evidence of disease, confirmed by pathology).

[0017] Figure 1B shows a graph depicting mean reflectance spectra before application of acetic acid and at various times following the application of acetic acid for NED tissue (no evidence of disease, confirmed by pathology).

[0018] Figure 2A shows a graph depicting mean fluorescence spectra before application of acetic acid and at various times following the application of acetic acid for CIN 2/3 tissue (grades 2 and/or 3 cervical intraepithelial neoplasia).

[0019] Figure 2B shows a graph depicting mean reflectance spectra before application of acetic acid and at various times following the application of acetic acid for CIN 2/3 tissue (grades 2 and/or 3 cervical intraepithelial neoplasia).

[0020] Figure 3A shows a graph depicting fluorescence intensity at three different wavelengths relative to pre-AA (fluorescence before application of acetic acid) as a function of time following application of acetic acid for NED tissue.

[0021] Figure 3B shows a graph depicting reflectance at three different wavelengths relative to pre-AA (reflectance before application of acetic acid) as a function of time following application of acetic acid for NED tissue.

[0022] Figure 3C shows a graph depicting fluorescence intensity at three different wavelengths relative to pre-AA (fluorescence before application of acetic acid) as a function of time following application of acetic acid for CIN 2/3 tissue.

[0023] Figure 3D shows a graph depicting reflectance at three different wavelengths relative to pre-AA (reflectance before application of acetic acid) as a function of time following application of acetic acid for CIN 2/3 tissue.

[0024] Figure 4A shows a graph depicting reflectance relative to pre-AA at 425 nm as a function of time following application of acetic acid for various tissue types.

[0025] Figure 4B shows a graph depicting fluorescence relative to pre-AA at 460 nm as a function of time following application of acetic acid for various tissue types.

[0026] Figure 5 shows a series of graphs depicting mean reflectance spectra for CIN 2/3 and non-CIN 2/3 (NED and CIN 1) tissues at a time prior to application of acetic acid, at a time corresponding to maximum whitening, and at a time corresponding to the latest time at which data was obtained.

[0027] Figure 6 shows a graph depicting the reflectance discrimination function spectra useful for differentiating between CIN 2/3 and non-CIN 2/3 (NED and CIN 1) tissues.

[0028] Figure 7 shows a graph depicting the performance of two LDA (linear discriminant analysis) models as applied to reflectance data obtained at various times following application of acetic acid; one of the models is based on data obtained between 60 and 80 seconds following application of acetic acid, and the other model is based on data obtained between 160 and 180 seconds following application of acetic acid.

[0029] Figure 8 shows a series of graphs depicting mean fluorescence spectra for CIN 2/3 and non-CIN 2/3 (NED and CIN 1) tissues at a time prior to application of acetic acid, at a

time corresponding to maximum whitening, and at a time corresponding to the latest time at which data was obtained.

[0030] Figure 9 shows a graph depicting the fluorescence discrimination function spectra useful for differentiating between CIN 2/3 and non-CIN 2/3 (NED and CIN 1) tissues.

[0031] Figure 10 shows a graph depicting the performance of two LDA (linear discriminant analysis) models as applied to fluorescence data obtained at various times following application of acetic acid; one of the models is based on data obtained between 60 and 80 seconds following application of acetic acid, and the other model is based on data obtained between 160 and 180 seconds following application of acetic acid.

[0032] Figure 11 shows a graph depicting the performance of three LDA models as applied to data obtained at various times following application of acetic acid.

[0033] Figure 12A shows a graph depicting the determination of an optimal time window for obtaining diagnostic optical data using an optical amplitude trigger.

[0034] Figure 12B shows a graph depicting the determination of an optimal time window for obtaining diagnostic data using a rate of change of mean reflectance signal trigger.

Description of the Illustrative Embodiment

[0035] The invention relates to methods for determining a characteristic of a tissue sample using spectral data and/or images obtained within an optimal window of time following the application of a chemical agent to the tissue sample. The invention provides methods of determining optimal windows of time. Similarly, the invention provides methods of determining criteria, based on a spectral amplitude or rate of amplitude change, for triggering the acquisition of an optical signal for classifying tissue. Finally, the invention comprises methods of diagnosing a tissue sample using spectral data and/or images obtained within an optimal window.

[0036] Application of the invention allows the diagnosis of regions of a tissue sample using various features of the time response of fluorescence and/or reflectance spectra following the application of a contrast agent such as acetic acid. For example, it is possible to diagnose a region of a tissue sample by determining a time at which a minimum value of fluorescence spectral intensity is reached following application of a contrast agent.

[0037] Methods of the invention are also used to analyze tissue samples, including cervical tissue, colorectal tissue, gastroesophageal tissue, urinary bladder tissue, lung tissue, or other tissue containing epithelial cells. The tissue may be analyzed *in vivo* or *ex vivo*, for example. Tissue samples are generally divided into regions, each having its own characteristic. This characteristic may be a state of health, such as intraepithelial neoplasia, mature and immature metaplasia, normal columnar epithelia, normal squamous epithelia, and cancer. Chemical contrast agents which are used in practice of the invention include acetic acid, formic acid, propionic acid, butyric acid, Lugol's iodine, Shiller's iodine, methylene blue, toluidine blue, indigo carmine, indocyanine green, fluorescein, and combinations comprising these agents. In

embodiments where acetic acid is used, concentrations between about 3 volume percent and about 6 volume percent acetic acid are typical, although in some embodiments, concentrations outside this range may be used. In one embodiment, a 5 volume percent solution of acetic acid is used as contrast agent.

[0038] Optical signals used in practice of the invention comprise, for example, fluorescence, reflectance, Raman, infrared, and video signals. Video signals comprise images from standard black-and-white or color CCD cameras, as well as hyperspectral imaging signals based on fluorescence, reflectance, Raman, infrared, and other spectroscopic techniques. For example, an embodiment comprises analyzing an intensity component indicative of the "whiteness" of a pixel in an image during an acetowhitening test.

[0039] A preferred embodiment uses optical signals obtained from tissue samples within optimal windows of time. Obtaining an optical signal may comprise actually acquiring a signal within an optimal window of time, or, of course, simply triggering the acquisition of an optical signal within an optimal window of time. The optimal window of time may account for a delay between the triggering of the acquisition of a signal, and its actual acquisition. An embodiment of the invention may comprise determining an optimal window of time in which to trigger the acquisition of an optical signal, as well as determining an optimal window of time in which to actually acquire an optical signal.

[0040] One embodiment comprises determining an optimum time window in which to obtain spectra from cervical tissue such that sites indicative of grades 2 and 3 cervical intraepithelial neoplasia (CIN 2/3) can be separated from non-CIN 2/3 sites. Non-CIN 2/3 sites include sites with grade 1 cervical intraepithelial neoplasia (CIN 1), as well as NED sites (which include mature and immature metaplasia, and normal columnar and normal squamous

epithelia). Alternately, sites indicative of high grade disease, CIN 2+, which includes CIN 2/3 categories, carcinoma in situ (CIS), and cancer, may be separated from non-high-grade-disease sites. In general, for any embodiment in which CIN 2/3 is used as a category for classification or characterization of tissue, the more expansive category CIN 2+ may be used alternatively. One embodiment comprises differentiating amongst three or more classification categories. Exemplary embodiments are described below and comprise analysis of the time response of diffuse reflectance and/or 337-nm fluorescence spectra of a set of reference tissue samples with regions having known states of health, as listed in the Appendix Table, to determine temporal characteristics indicative of the respective states of health. These characteristics are then used in building a model to determine a state of health of an unknown tissue sample. Other embodiments comprise analysis of fluorescence spectra using other excitation wavelengths, such as 380nm and 460nm, for example.

[0041] While the invention is particularly shown and described herein with reference to specific examples and specific embodiments, it should be understood by those skilled in the art that various changes in form and detail may be made therein without departing from the spirit and scope of the invention.

Example 1: Analysis of the temporal evolution of spectral data from reference samples with known states of health.

[0042] Diffuse reflectance and/or 337-nm fluorescence emission spectra are taken from cervical tissue samples that are categorized as CIN 2/3 (having grades 2 and/or 3 cervical intraepithelial neoplasia), CIN 1 and NED (no evidence of disease, confirmed by pathology, including normal squamous tissue, normal columnar tissue, immature metaplasia tissue, and mature metaplasia tissue). All spectra are filtered then placed in the time bins indicated in

Table 1. Data affected by artifacts such as glare, shadow, or obstructions may be removed and/or compensated for by using the technique disclosed in the co-owned U.S. patent application entitled, "Method and Apparatus for Identifying Spectral Artifacts," filed on September 13, 2002, and identified by attorney docket number MDS-033, the contents of which are hereby incorporated by reference. Means spectra and standard deviations are calculated for the spectra in each time bin. Although not shown in this example, some embodiments use spectral and/or image data obtained at times greater than 180 s following application of contrast agent.

Table 1: Time bins for which means spectra are calculated in an exemplary embodiment

Bin	Time after application of Acetic Acid (s)
1	$t \leq 0$
2	$0 < t \leq 40$
3	$40 < t \leq 60$
4	$60 < t \leq 80$
5	$80 < t \leq 100$
6	$100 < t \leq 120$
7	$120 < t \leq 140$
8	$140 < t \leq 160$
9	$160 < t \leq 180$
10	$t > 180$

[0043] Figures 1A, 1B, 2A, and 2B show mean fluorescence and reflectance spectra for exemplary healthy tissue (NED tissue – no evidence of disease, confirmed by pathology) and CIN 2/3 (grades 2 and/or 3 cervical intraepithelial neoplasia) tissue samples. These figures demonstrate the temporal effect of acetic acid on the spectral data. In the application of one

embodiment, one or more characteristics of the time responses shown in Figures 1A, 1B, 2A, and 2B are determined. Subsequently, the time response of a sample of unknown type is obtained, and the sample is then diagnosed according to one or more features of the response, compared against those of the known sample set.

[0044] Figure 1A shows a graph 102 depicting mean fluorescence spectra for each of the 10 time bins 108 of Table 1 for NED tissue (no evidence of disease, confirmed by pathology). Mean fluorescence intensity (relative counts/μJ) 104 is plotted as a function of wavelength (nm) 106 for each time bin shown in the legend 108. The curve corresponding to the first time bin 110 is a graph of the mean fluorescence intensity as a function of wavelength for data collected prior to acetic acid application, and the curve corresponding to the last time bin 128 is a graph of the mean fluorescence intensity as a function of wavelength for data collected at times greater than 180 seconds (with an average of about 210 seconds). Each of the curves in between (112, 114, 116, 118, 120, 122, 124, 126) is a graph of the mean fluorescence intensity as a function of wavelength for data collected in the respective time bin shown in the legend 108. The value of N shown in the legend 108 beside each curve denotes the number of spectra that are in the respective time bin for this particular embodiment.

[0045] Figure 1B shows a graph 150 depicting mean reflectance spectra for each of the 10 time bins 108 of Table 1 for NED tissue (no evidence of disease, confirmed by pathology). Mean reflectance 152 is plotted as a function of wavelength (nm) 106 for each time bin shown in the legend 108. The curve corresponding to the first time bin 154 is a graph of the mean reflectance as a function of wavelength for data collected prior to acetic acid application, and the curve corresponding to the last time bin 172 is a graph of the mean reflectance as a function of wavelength for data collected at times greater than 180 seconds (with an average

of about 210 seconds). Each of the curves in between (156, 158, 160, 162, 164, 166, 168, 170) is a graph of the mean reflectance as a function of wavelength for data collected in the respective time bin shown in the legend 108. The value of N shown in the legend 108 beside each curve denotes the number of spectra that are in the respective time bin for this particular embodiment.

[0046] Figure 2A shows a graph 202 depicting mean fluorescence spectra for each of the 10 time bins 204 of Table 1 for CIN 2/3 tissue (grades 2 and/or 3 cervical intraepithelial neoplasia). Mean fluorescence intensity (relative counts/μJ) 104 is plotted as a function of wavelength (nm) 106 for each time bin shown in the legend 204. The curve corresponding to the first time bin 206 is a graph of the mean fluorescence intensity as a function of wavelength for data collected prior to acetic acid application, and the curve corresponding to the last time bin 224 is a graph of the mean fluorescence intensity as a function of wavelength for data collected at times greater than 180 seconds (with an average of about 210 seconds). Each of the curves in between (208, 210, 212, 214, 216, 218, 220, 220) is a graph of the mean fluorescence intensity as a function of wavelength for data collected in the respective time bin shown in the legend 204. The value of N shown in the legend 204 beside each curve denotes the number of spectra that are in the respective time bin for this particular embodiment.

[0047] Figure 2B shows a graph 250 depicting mean reflectance spectra for each of the 10 time bins 204 of Table 1 for CIN 2/3 tissue (grades 2 and/or 3 cervical intraepithelial neoplasia). Mean reflectance 152 is plotted as a function of wavelength (nm) 106 for each time bin shown in the legend 204. The curve corresponding to the first time bin 254 is a graph of the mean reflectance as a function of wavelength for data collected prior to acetic acid application, and the curve corresponding to the last time bin 272 is a graph of the mean

reflectance as a function of wavelength for data collected at times greater than 180 seconds (with an average of about 210 seconds). Each of the curves in between (256, 258, 260, 262, 264, 266, 268, 270) is a graph of the mean reflectance as a function of wavelength for data collected in the respective time bin shown in the legend 204. The value of N shown in the legend 204 beside each curve denotes the number of spectra that are in the respective time bin for this particular embodiment.

Example 2: Analysis of optical kinetic data from reference samples with known states of health.

[0048] Data from Figures 1A, 1B, 2A, and 2B are further analyzed as shown in Figures 3A, 3B, 3C, and 3D. Figure 3A shows a graph 302 depicting the time response of fluorescence intensity relative to pre-AA (fluorescence prior to application of acetic acid) 304 of NED tissue at 390, 460 and 600 nm wavelengths following application of acetic acid. Figure 3B shows a graph 320 depicting the time response of reflectance relative to pre-AA 322 for NED tissue at 425, 500, and 630 nm wavelengths following application of acetic acid. Figure 3C shows a graph 350 depicting the time response of fluorescence intensity relative to pre-AA 304 of CIN 2/3 tissue at 390, 460, and 600 nm wavelengths following application of acetic acid. Figure 3D shows a graph 370 depicting the time response of reflectance relative to pre-AA 322 for CIN 2/3 tissue at 425, 500, and 630 nm wavelengths following application of acetic acid.

[0049] The fluorescence intensity in the NED group continues to drop over the time period studied while some recovery is seen in the fluorescence intensity of the CIN 2/3 group. Figure 3A reveals a continuous drop in fluorescence for the NED group over the measurement period at the three wavelengths. In contrast, Figure 3C shows partial recovery at all three

wavelengths for CIN 2/3 tissue. Each of the curves representing CIN 2/3 tissue labeled 352, 354, and 356 in Figure 3C demonstrates a generalized local minimum at a time from about 70 to about 130 seconds following application of acetic acid, whereas each of the curves representing NED tissue labeled 310, 312, and 314 in Figure 3A does not show such a local minimum.

[0050] The fluorescence and reflectance kinetics are similar for the CIN 2/3 group but differ for the NED group. Partial recovery (return toward initial condition) is noted in both the reflectance and the fluorescence curves at all 3 wavelengths for CIN 2/3 tissue, as shown in the curves labeled 352, 354, 356, 372, 374, and 376 in Figure 3C and Figure 3D. However, partial recovery is noted only in the reflectance curves for NED tissue (curves 326, 328, and 330 of Figure 3B), while the NED fluorescence intensities continue to drop (curves 310, 312, and 314 of Figure 3A).

[0051] The magnitude of change in the time response of reflectance and fluorescence data following application of acetic acid is different between the CIN 2/3 group and the NED group. The relative maximum change in reflectivity at about 425 nm is about twice as large for CIN 2/3 (i.e. line segment 274 in Figure 2B) compared to non-CIN (i.e. line segment 174 in Figure 1B), while the maximum change for fluorescence is approximately equivalent for CIN 2/3 and non-CIN samples. Here, the magnitude of change in the reflectance signal depends on tissue type while the magnitude of change in the fluorescence signal does not depend on tissue type.

[0052] The time to reach the maximum change in fluorescence is delayed for NED spectra. This is shown by comparing curves 310, 312, and 314 of Figure 3A with curves 352, 354, and

356 of Figure 3C. It is therefore possible, for example, to use the time required to reach a minimum value of fluorescence spectral intensity to distinguish CIN 2/3 from NED samples.

[0053] The fluorescence line-shape changes with time post acetic acid, particularly at later times where a valley at about 420 nm and a band at about 510 nm become more distinct. The valley at about 420 nm is shown in Figure 1A at reference number 130 and in Figure 2A at reference number 226, while the band at about 510 nm can be seen in Figure 1A at reference number 132 and in Figure 2A at reference number 228. One explanation for this change is that collagen and NADH decrease tissue fluorescence and FAD increases tissue fluorescence. Upon introduction of a change in pH from 7 to 3.5, the fluorescence intensity of NADH decreases by a factor of two while FAD increases six-fold. Increased scattering in the epithelial layer would decrease the contribution of collagen fluorescence from the submucosal layer. Characterization of such changes in spectral curve shape is useful, for example, in distinguishing tissue types.

[0054] In one embodiment, an optimal window for obtaining spectral and/or image data is a period of time in which there is a peak "whitening" as seen in image and/or reflectance data. In another embodiment, an optimal window is a period of time in which there is a peak "darkening" of fluorescence of the tissue. Still another embodiment uses a subset of the union of the two optimal windows above. Figures 1A, 1B, 2A, 2B, 3A, 3B, 3C, and 3D demonstrate "whitening" of reflectance and "darkening" of fluorescence as a function of wavelength and time following application of acetic acid. The maximum change observed in the CIN 2/3 group is determined from the data shown in Figures 2A, 2B, 3C, and 3D. Here, the peak "darkening" of the fluorescence data lags peak "whitening" of the reflectance data. From the reflectance data, the window for peak whitening lies between about 30 s and about 110 s

following the application of acetic acid with a maximum at about 70 s. In one embodiment, the peak whitening window lies between about 30 s and about 130 s; and in another embodiment from about 20 s to about 180 s. For fluorescence, the peak "darkening" window lies between about 50 s and about 150 s with a minimum at about 80 s. In one embodiment, the peak darkening window lies between about 60 s and about 220 s. Peak "whitening" for the non-CIN reflectance spectra is less intense but similar in shape to that found for CIN 2/3. Peak darkening in non-CIN fluorescence appears later than in CIN 2/3 fluorescence.

[0055] Figures 4A and 4B depict the influence of acetic acid on reflectance and fluorescence intensities at about 425 nm and about 460 nm, respectively, for various reference tissue classes. These classes include CIN 2/3 (curves 406 and 454), CIN 1 (curves 408 and 456), metaplasia TT016 and TT017 (curves 410 and 458), normal columnar TT022 (curves 412 and 460) and normal squamous TT025 (curves 414 and 462) tissues, as shown in Figures 4A and 4B. In general, the reflectance curves of Figure 4A show some distinct differences with tissue type, with CIN 2/3 tissue (curve 406) having the largest change. Columnar epithelial tissue (curve 412) shows rapid relatively intense whitening followed by rapid recovery while squamous epithelial tissue (curve 414) has a weak, slow response with very little recovery. Metaplastic tissues (curve 410) and tissue with CIN 1 (curve 408) have similar behavior with a relatively fast increase and decay. The acetowhitening response of all tissue groups ride on top of a slowing, increasing background, thereby suggesting a secondary response to acetic acid. This secondary response is most distinct in the CIN 1 group and appears to be the predominant response in the normal squamous group.

[0056] The magnitude of the acetodarkening effect for fluorescence is similar independent of tissue type, as shown in Figure 4B. The time to reach a minimum fluorescence is different

for different tissue classes, with normal squamous tissue (curve 462) having the slowest response and normal columnar tissue (curve 460) having the fastest response. The response for CIN 2/3 (curve 454), CIN 1 (curve 456), and metaplastic tissues (curve 458) are very similar. There is partial recovery from the acetic acid effect in the CIN 2/3 group (curve 454).

Example 3: Using a discrimination function to determine optimal windows for obtaining diagnostic optical data.

[0057] An embodiment of the invention comprises determining an optimum window for obtaining diagnostic spectral data using fluorescence and/or reflectance time-response data as shown in the above figures, and as discussed above. In one embodiment, an optimum window is determined by tracking the difference between spectral data of various tissue types using a discrimination function.

[0058] In one embodiment, the discrimination function shown below in Equation (1) is used to extract differences between tissue types:

$$D(\lambda) = \frac{\mu(test(\lambda))_{NEDPATH1} - \mu(test(\lambda))_{CIN23ALL}}{\sqrt{\sigma^2(test(\lambda))_{NEDPATH1} + \sigma^2(test(\lambda))_{CIN23ALL}}} \quad (1)$$

The quantity μ corresponds to the mean optical signal and σ corresponds to the standard deviation. In one embodiment, the optical signal includes diffuse reflectance. In another embodiment, the optical signal includes 337-nm fluorescence emission spectra. Other embodiments use fluorescence emission spectra at another excitation wavelength such as 380 nm and 460 nm. In still other embodiments, the optical signal is a video signal, Raman signal, or infrared signal. Some embodiments comprise using difference spectra calculated between

different phases of acetowhitening, using various normalization schema, and/or using various combinations of spectral data and/or image data as discussed above.

[0059] One embodiment comprises developing linear discriminant analysis models using spectra from each time bin as shown in Table 1. Alternatively, nonlinear discriminant analysis models may be developed. Generally, models are trained using reflectance and fluorescence data separately, although some embodiments comprise use of both data types to train a model. In exemplary embodiments discussed below, reflectance and fluorescence intensities are down-sampled to one value every 10 nm between 360 and 720 nm. A model is trained by adding and removing intensities in a forward manner, continuously repeating the process until the model converges such that additional intensities do not appreciably improve tissue classification. Testing is performed by a leave-one-spectrum-out jack-knife process.

[0060] Figure 5 shows the difference between the mean reflectance spectra for non-CIN 2/3 tissues (including CIN 1 and NED tissues) and CIN 2/3 tissues at three times – at a time prior to the application of acetic acid (graph 502), at a time corresponding to maximum whitening (graph 520, about 60 – 80 seconds post-AA), and at a time corresponding to the latest time period in which data was obtained (graph 550, about 160 - 180 seconds post-AA). Here, the time corresponding to maximum whitening was determined from reflectance data, and occurs between about 60 seconds and 80 seconds following application of acetic acid. In the absence of acetic acid, the reflectance spectra for CIN 2/3 (curve 510 of graph 502 in Figure 5) are on average lower than non-CIN 2/3 tissue (curve 508 of graph 502 in Figure 5). Following the application of acetic acid, a reversal is noted with CIN 2/3 tissues having higher reflectance than the other tissues. The reflectance of CIN 2/3 and non-CIN 2/3 tissues increase with acetic acid, with CIN 2/3 showing a larger relative percent change (compare curves 522 and

524 of graph 520 in Figure 5). From about 160 s to about 180 s following acetic acid, the reflectance of CIN 2/3 tissue begins to return to the pre-acetic acid state, while the reflectance of the non-CIN 2/3 group continues to increase (compare curves 552 and 554 of graph 550 in Figure 5)

[0061] In one embodiment, discrimination function 'spectra' are calculated from the reflectance spectra of CIN 2/3 and non-CIN 2/3 tissues shown in Figure 5. In one example, discrimination function spectra comprise values of the discrimination function in Equation (1) determined as a function of wavelength for sets of spectral data obtained at various times. Figure 6 shows a graph 602 depicting the discrimination function spectra evaluated using the diffuse reflectance data of Figure 5 obtained prior to application of acetic acid, and at two times post-AA. Curve 608 corresponds to the discrimination function 604 evaluated as a function of wavelength 606 using non-CIN 2/3 data and CIN 2/3 data obtained prior to application of acetic acid. Curve 610 corresponds to the discrimination function 604 evaluated as a function of wavelength 606 using non-CIN 2/3 data and CIN 2/3 data obtained between about 60 and about 80 seconds after application of acetic acid; and curve 612 corresponds to the discrimination function 604 evaluated as a function of wavelength 606 using non-CIN 2/3 data and CIN 2/3 data obtained between about 160 and about 180 seconds after application of acetic acid. Distinguishing between CIN 2/3 and non-CIN 2/3 tissues using reflectance data is improved with the application of acetic acid. Here, the largest differences (for example, the largest absolute values of discrimination function) are found from data measured from about 60 s to about 80 s post-acetic acid (curve 610), and these agree with the differences seen in the mean reflectance spectra of Figure 5 (curves 522 and 524 of graph 520 in Figure 5).

[0062] Performing multivariate linear regression analysis addresses wavelength interdependencies in the development of a classification model. An application of one embodiment comprises classifying data represented in the CIN 2/3, CIN 1, and NED categories in the Appendix Table into CIN 2/3 and non-CIN 2/3 categories by using classification models developed from the reflectance data shown in Figure 5. Here, reflectance intensities are down-sampled to one about every 10 nm between about 360 nm and about 720 nm. The model is trained by adding intensities in a forward-stepped manner. Testing is performed with a leave-one-spectrum-out jack-knife process. The result of this analysis shows which wavelengths best separate CIN 2/3 from non-CIN 2/3, as shown in Table 2 for an exemplary embodiment.

Table 2: Forwarded selected best reflectance wavelengths for classifying CIN 2/3 from non-CIN 2/3 spectra obtained at different times pre and post-AA.

Time from AA (s)	LDA Model Input Wavelengths (nm)	Accuracy
-30	370 400 420 440 530 570 590 610	66
30	420 430 450 600	74
50	360 400 420 430 580 600	74
70	360 370 420 430 560 580 600	74
90	360 420 430 540 590	73
110	360 440 530 540 590	71
130	360 420 430 540 590	71
150	370 400 430 440 540 620 660 690 720	72
170	490 530 570 630 650	75

[0063] The two best models for separating CIN 2/3 and non-CIN 2/3 for this embodiment include the model using reflectance data obtained at peak CIN 2/3 whitening (from about 60s to about 80s) and the model using reflectance data from the latest time measured (from about 160s to about 180s post acetic acid). The first model uses input wavelengths between about

360 and about 600 nm, while the second model uses more red-shifted wavelengths between about 490 and about 650 nm. This is consistent with the behavior of the discrimination function spectra shown in Figure 6.

[0064] Figure 7 demonstrates one method of determining an optimal window for obtaining reflectance spectral data in the diagnosis of the state of health of a region of a sample as CIN 2/3 or non-CIN 2/3. Figure 7 shows a graph 702 depicting the performance of the two LDA models described in Table 2 above as applied to reflectance spectral data obtained at various times following application of acetic acid 706. Curve 710 in Figure 7 is a plot of the diagnostic accuracy of the LDA model based on reflectance spectral data obtained between about 60 and about 80 seconds ("peak whitening model") as applied to reflectance spectra from the bins of Table 1, and curve 712 in Figure 7 is a plot of the diagnostic accuracy of the LDA model based on reflectance spectral data obtained between about 160 and about 180 seconds, as applied to reflectance spectra from the bins of Table 1. For the peak-whitening model, the highest accuracy was obtained at about 70 s, while accuracies greater than 70% were obtained with spectra collected in a window between about 30s and about 130s. The 160-180 s model had a narrower window around 70 s, but performs better at longer times.

[0065] Figure 8 shows the difference between the mean 337-nm fluorescence spectra for non-CIN 2/3 tissues (including CIN 1 and NED tissues) and CIN 2/3 tissues at three times – at a time prior to application of acetic acid (graph 802), at a time corresponding to maximum whitening (graph 820, about 60 to about 80 seconds post-AA), and at a time corresponding to the latest time period in which data was obtained (graph 850, about 160 to about 180 seconds post-AA). The time corresponding to maximum whitening was determined from reflectance data, and occurs between about 60 seconds and 80 seconds following application of acetic

acid. In the absence of acetic acid, the fluorescence spectra for CIN 2/3 tissue (curve 812 of graph 802 in Figure 8) and for non-CIN 2/3 tissue (curve 810 of graph 802 in Figure 8) are essentially equivalent with a slightly lower fluorescence noted around 390 nm for CIN 2/3 sites. Following the application of acetic acid, the fluorescence of CIN 2/3 and non-CIN 2/3 tissues decrease, with CIN 2/3 showing a larger relative percent change (compare curves 824 and 822 of graph 820 in Figure 8). From about 160s to about 180 s following acetic acid application, the fluorescence of CIN 2/3 tissue shows signs of returning to the pre-acetic acid state while the fluorescence of the non-CIN 2/3 group continues to decrease (compare curves 854 and 852 of graph 850 in Figure 8).

[0066] In one embodiment, discrimination function 'spectra' are calculated from the fluorescence spectra of CIN 2/3 and non-CIN 2/3 tissues shown in Figure 8. In one example, discrimination function spectra comprise values of the discrimination function in Equation (1) determined as a function of wavelength for sets of spectral data obtained at various times. Figure 9 shows a graph 902 depicting the discrimination function spectra evaluated using the fluorescence data of Figure 8 obtained prior to application of acetic acid, and at two times post-AA. Curve 908 corresponds to the discrimination function 904 evaluated as a function of wavelength 906 using non-CIN 2/3 data and CIN 2/3 data obtained prior to application of acetic acid. Curve 910 corresponds to the discrimination function 904 evaluated as a function of wavelength 906 using non-CIN 2/3 data and CIN 2/3 data obtained between about 60 and about 80 seconds after application of acetic acid; and curve 912 corresponds to the discrimination function 904 evaluated as a function of wavelength 906 using non-CIN 2/3 data and CIN 2/3 data obtained between about 160 and about 180 seconds after application of acetic acid. Distinguishing between CIN 2/3 and non-CIN 2/3 tissues using fluorescence data

is improved with the application of acetic acid. Here, the largest absolute values are found from data measured within the range of about 160-180 s post-acetic acid (curve 912), and these agree with the differences seen in the mean fluorescence spectra of Figure 8 (curves 852 and 854 of graph 850 in Figure 8).

[0067] Performing multivariate linear regression analysis addresses wavelength interdependencies in the development of a classification model. An application of one embodiment comprises classifying data represented in the CIN 2/3, CIN 1, and NED categories in the Appendix Table into CIN 2/3 and non-CIN 2/3 categories by using classification models developed from the fluorescence data shown in Figure 8. Fluorescence intensities are down-sampled to one about every 10 nm between about 360 and about 720 nm. The model is trained by adding intensities in a forward manner. Testing is performed by a leave-one-spectrum-out jack-knife process. The result of this analysis shows which wavelengths best separate CIN 2/3 from non-CIN 2/3, as shown in Table 3 for an exemplary embodiment.

Table 3: Forwarded selected best 337-nm fluorescence wavelengths for classifying CIN 2/3 from non-CIN 2/3 spectra obtained at different times pre and post-AA.

Time from AA (s)	LDA Model Input Wavelengths (nm)	Accuracy
-30	380, 430, 440, 610, 660, 700, 710	61
30	370, 380, 390, 640	61
50	410	54
70	360, 390, 490, 580, 590, 670	63
90	370, 380, 420, 460, 500, 560, 660	64
110	360, 390, 400, 710	51
130	370	53
150	370, 380, 440, 620, 640, 700	65
170	370, 480, 510, 570, 600, 700, 720	76

[0068] The two best models for separating CIN 2/3 and non-CIN 2/3 for this embodiment include the model using data obtained at peak CIN 2/3 whitening (60-80 s) and the model using data at the latest time measured (160-180 s post acetic acid). The first model uses input wavelengths between about 360 and about 670 nm, while the second model uses wavelengths between about 370 and about 720 nm. This is consistent with the discrimination function spectra shown in Figure 9.

[0069] Figure 10 demonstrates one method of determining an optimal window for obtaining fluorescence spectral data in the diagnosis of the state of health of a region of a sample as CIN 2/3 or non-CIN 2/3. Figure 10 shows a graph 1002 depicting the performance of the two LDA models described in Table 3 above as applied to fluorescence spectral data obtained at various times following application of acetic acid 1006. Curve 1010 in Figure 10 is a plot of the diagnostic accuracy of the LDA model based on fluorescence spectral data obtained between about 60 and about 80 seconds ("peak whitening model") as applied to fluorescence spectra from the bins of Table 1, and curve 1012 in Figure 10 is a plot of the diagnostic accuracy of the LDA model based on fluorescence spectral data obtained between about 160

and about 180 seconds, as applied to fluorescence spectra from the bins of Table 1. The accuracies of these models vary depending on when the fluorescence spectra are recorded relative to the application of acetic acid, as shown in Figure 10. The predictive ability of the fluorescence models in Figure 10 tend to be less than that of the reflectance models in Figure 7. Accuracies greater than 70% are obtained with spectra collected after about 160 seconds post-AA.

[0070] Another embodiment comprises classifying data represented in the CIN 2/3, CIN 1, and NED categories in the Appendix Table into CIN 2/3 and non-CIN 2/3 categories by using fluorescence divided by diffuse reflectance spectra. Models are developed based on time post acetic acid. Ratios of fluorescence to reflectance are down-sampled to one every 10 nm between about 360 and about 720 nm. The model is trained by adding intensities in a forward manner. Testing is performed by a leave-one-spectrum-out jack-knife process. For this analysis, the model is based on intensities at about 360, 400, 420, 430, 560, 610, and 630 nm. In general, the results are slightly better than a model based on fluorescence alone. Improved performance is noted from spectra acquired at about 160 s post acetic acid.

[0071] Figure 11 shows a graph 1102 depicting the accuracy of three LDA models as applied to spectral data obtained at various times following application of acetic acid. Curve 1110 in Figure 11 is a plot of the diagnostic accuracy of the LDA model based on reflectance spectral data obtained between about 60 and about 80 seconds ("peak whitening model"), also shown as curve 710 in Figure 7. Curve 1112 in Figure 11 is a plot of the diagnostic accuracy of the LDA model based on fluorescence spectral data obtained between about 60 and about 80 seconds ("peak whitening model"), also shown as curve 1010 in Figure 10. Curve 1114 in

Figure 11 is a plot of the diagnostic accuracy of the LDA model based on fluorescence intensity divided by reflectance, as described in the immediately preceding paragraph.

[0072] The exemplary embodiments discussed above demonstrate that the ability to distinguish between non-CIN 2/3 and CIN 2/3 fluorescence and reflectance spectra is improved with the application of acetic acid or other contrast agent. For the peak-whitening LDA model using reflectance data, the highest accuracy for the exemplary applications of the embodiments discussed herein is obtained at about 70 s following introduction of acetic acid, while accuracies greater than about 70% are obtained with spectra collected in a window between about 30 s and about 130 s. The predictive ability of the fluorescence models in the examples above tend to be less than that of the reflectance models for the examples discussd above. Accuracies greater than 70% are obtained with fluorescence at times greater than about 160 s post acetic acid. The intensity of fluorescence continuously drop over the measurement period in the non-CIN groups while partial recovery occurs at all 3 emission wavelengths in the CIN 2/3 group, suggesting that fluorescence spectral data obtained at times greater than about 180 s is useful in diagnosing CIN 2/3.

Example 4: Other kinetics-based approaches for obtaining diagnostic optical data within an optimal window.

[0073] As an alternative to the techniques discussed above, other kinetics-based approaches may be used to determine classification models and, hence, corresponding optimum windows for classification of tissue samples. The time response of fluorescence intensity or the time response of reflectance following application of contrast agent, as shown in Figure 3 and Figure 4, may be curve-fitted to determine one or more parameters sensitive to a curve feature of interest. For example, a parameter sensitive to a local minimum may be determined for a given set of fluorescence response data. In one embodiment, a parameter is determined by curve-fitting fluorescence time response data to a sigmoidal function. Values of the parameter and/or goodness-of-fit data are then used to develop a statistical model for classifying a sample in terms of a characteristic of the sample, such as its state of health. The model is built using reference data with known states of health. Then, the time response of spectral intensity of a test sample with unknown state of health following application of a contrast agent is obtained. By curve-fitting this response data, values of the indicated parameter(s) may be obtained, and the model may be used to either directly determine the characteristic of the test sample, or to indicate an optimal window in which spectral data should be obtained and used to accurately classify the tissue. In one embodiment, the parameter determined by curve-fitting spectral time response curves is not used directly to classify the tissue, but is used to determine an optimal window. The parameter indicates a window of time in which one or more complete sets of spectral and/or video data should be obtained for accurate diagnosis of the tissue.

Example 5: Using a relative change or rate-of-change trigger to obtain diagnostic optical data

[0074] An embodiment of the invention comprises determining and using a relative amplitude change and/or rate of amplitude change as a trigger for obtaining diagnostic optical data from a sample. The trigger can also be used to determine an optical window of time for obtaining such diagnostic optical data. By using statistical and/or heuristic methods such as those discussed herein, it is possible to relate more easily-monitored relative changes or rates-of-change of one or more optical signals from a tissue sample to corresponding full spectrum signals that can be used in characterizing the state of health of a given sample. For example, by performing a discrimination function analysis, it may be found for a given tissue type that when the relative change in reflectance at a particular wavelength exceeds a threshold value, the corresponding full-spectrum reflectance can be obtained and then used to accurately classify the state of health of the tissue. In addition, the triggers determined above may be converted into optimal time windows for obtaining diagnostic optical data from a sample.

[0075] Figure 12A shows how an optical amplitude trigger can be used to determine an optimal time window for obtaining diagnostic optical data. The graph 1200 in Figure 12A plots the normalized relative change of mean reflectance signal 1202 from tissue samples with a given state of health as a function of time following application of acetic acid 1204. The mean reflectance signal determined from CIN 1, CIN 2, and Metaplasia samples are depicted in Figure 12A by curves 1210, 1208, and 1212, respectively. Here, it was determined that when the normalized relative change of mean reflectance reaches or exceeds 0.75, the image intensity data and/or the full reflectance and/or fluorescence spectrum for a given sample is most indicative of a given state of health of a sample. Thus, for CIN 2 samples, for example, this corresponds to a time period between t_1 and t_2, as shown in the graph 1200 of Figure 12A.

Therefore, spectral and/or image data obtained from a tissue sample between t_1 and t_2 seconds following application of acetic acid can be used in accurately determining whether or not CIN 2 is indicated for that sample. In one embodiment, the relative change of reflectance of a tissue sample at one or more given wavelengths is monitored, and when that relative change is greater than or equal to the 0.75 threshold, more comprehensive spectral and/or image data is obtained from the sample for purposes of characterizing whether or not the sample is indicative of CIN 2. Figure 12A demonstrates the use of a threshold value of relative optical signal change. In another embodiment, a predetermined range of values of the relative optical signal change is used such that when the relative signal change falls within the predetermined range of values, additional spectral and/or image data is captured in order to characterize the sample.

[0076]

Figure 12B shows how a rate-of-change of optical amplitude trigger can be used to determine an optimal time window for obtaining diagnostic optical data. The graph 1250 of Figure 12B plots the slope of mean reflectance signal 1252 from tissue samples with a given state of health as a function of time following application of acetic acid 1204. The slope of mean reflectance is a measure of the rate of change of the mean reflectance signal. The rate of change of mean reflectance determined from CIN 1, CIN 2, and Metaplasia samples are depicted in Figure 12B by curves 1258, 1256, and 1260, respectively. Here, it was determined that when the absolute value of the slope has an absolute value less than or equal to 0.1, for example, in the vicinity of maximum reflectance, the image intensity data and/or the full reflectance and/or fluorescence spectrum for a given sample is most indicative of a given state of health of a sample. Thus, for CIN 2 samples, for example, this corresponds to a

time period between t_1 and t_2 as shown in the graph 1250 of Figure 12B. Therefore, spectral and/or image data obtained from a tissue sample between t_1 and t_2 seconds following application of acetic acid can be used in accurately determining whether or not CIN 2 is indicated for that sample. In one embodiment, the rate of change of reflectance of a tissue sample is monitored at one or more given wavelengths, and when that rate of change has an absolute value less than or equal to 0.1, more comprehensive spectral and/or image data is obtained from the sample for purposes of characterizing whether or not the sample is indicative of CIN 2. Figure 12B demonstrates use of a range of values of rate of optical signal change. Other embodiments use a single threshold value.

Example 6: Using fluorescence, reflectance and/or image time response data to diagnose regions of tissue

[0077] The figures discussed herein include time-response fluorescence and reflectance data obtained following application of a contrast agent to tissue. In addition to an acetowhitening effect observed in the reflectance data, an "acetodarkening" effect is observed in the fluorescence data. For example, the fluorescence intensity of diseased regions decreases to a minimum at about 70 s to about 130 s following application of acetic acid. Thus, the presence of a minimum fluorescence intensity within this window of time, as well as the accompanying increase in fluorescence from this minimum, may be used to indicate disease. An embodiment of the invention comprises a method of identifying a characteristic of a region of a tissue sample including applying a contrast agent to a region of a tissue sample, obtaining at least two values of fluorescence spectral intensity corresponding to the region, determining whether the fluorescence spectral intensity corresponding to the region increases after a predetermined time following the applying step, and identifying a characteristic of the region based at least in part on the determining step. In an embodiment, the obtaining step comprises

obtaining a fluorescence spectral intensity signal corresponding to the region as a function of time following the applying step. In an embodiment, the method further comprises determining whether the fluorescence spectral intensity corresponding to the region decreases following the applying step, then increases after the predetermined time. In an embodiment, the predetermined time is about 80 seconds.

[0078] An embodiment comprises a method of identifying a characteristic of a region of a tissue sample comprising applying a contrast agent to a region of a tissue sample, obtaining a fluorescence spectral intensity signal from the region of the tissue sample, determining an elapsed time following the applying step at which the fluorescence spectral intensity signal has a minimum value, and identifying a characteristic of the region based at least in part on the elapsed time.

[0079] An embodiment comprises a method of identifying a characteristic of a region of a tissue sample comprising applying a contrast agent to a region of a tissue sample, obtaining a reflectance signal from the region of the tissue sample, determining a change in reflectance spectral intensity corresponding to the region of the tissue sample following the applying step, and identifying a characteristic of the region based at least in part on the change in reflectance spectral intensity. In an embodiment, the change in reflectance spectral intensity corresponding to the region comprises a change relative to an initial condition of the region.

[0080] An embodiment comprises a method of identifying a characteristic of a region of a tissue sample comprising applying a contrast agent to a region of a tissue sample, obtaining an optical signal from the region of the tissue sample, determining a rate of change of the optical signal corresponding to the region of the tissue sample, and identifying a characteristic of the region based at least in part on the rate of change. In an embodiment, the optical signal

comprises fluorescence spectral intensity at a given wavelength. In an embodiment, the optical signal comprises reflectance spectral intensity at a given wavelength.

[0081] An embodiment comprises a method of identifying a characteristic of a region of a tissue sample comprising applying a contrast agent to a region of a tissue sample, obtaining a fluorescence signal from the region of the tissue sample, obtaining a reflectance signal from the region of the tissue sample, and identifying a characteristic of the region based at least in part on the fluorescence signal and the reflectance signal.

[0082] An embodiment comprises obtaining an optical signal from 499 regions, each region having a diameter of approximately 1 mm, covering an area of tissue about 25 mm in diameter. An embodiment may also comprise obtaining a video image of about 480 by about 560 pixels covering the same 25-mm diameter area of tissue.

Appendix Table: Number of spectra (number of subjects) for each tissue class in each time bin for exemplary embodiments discussed herein.

Time	CIN 2/3	CIN 1	Metaplasia	TT_022[1]	TT_025[1]	NEDPath1[1]
$t \leq 0$	451 (62)	202 (46)	329 (77)	202 (56)	294 (70)	816 (186)
$0 < t \leq 40$	118 (21)	72 (14)	147 (33)	51 (14)	113 (22)	307 (64)
$40 < t \leq 60$	300 (47)	135 (31)	255 (58)	116 (32)	230 (51)	597 (133)
$60 < t \leq 80$	375 (54)	162 (39)	300 (68)	179 (42)	262 (61)	731 (157)
$80 < t \leq 100$	455 (60)	195 (42)	308 (70)	190 (49)	263 (64)	752 (167)
$100 < t \leq 120$	446 (60)	209 (45)	328 (76)	208 (52)	284 (68)	811 (178)
$120 < t \leq 140$	303 (44)	135 (30)	200 (48)	165 (43)	185 (51)	545 (129)
$140 < t \leq 160$	130 (18)	82 (17)	75 (19)	96 (23)	66 (21)	232 (53)
$160 < t \leq 180$	53 (9)	50 (9)	34 (9)	38 (12)	19 (6)	91 (24)
$t > 180$	14 (3)	26 (3)	33 (6)	23 (6)	30 (5)	86 (15)

[1] TT 022 = Normal columnar tissue; TT 025 = Normal squamous tissue; NEDPath1 = NED = Metaplasia, TT_022, and TT_025.

Equivalents

[0093] While the invention has been particularly shown and described with reference to specific preferred embodiments, it should be understood by those skilled in the art that various changes in form and detail may be made therein without departing from the spirit and scope of the invention as defined by the appended claims.

[0094] What is claimed is: